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Description

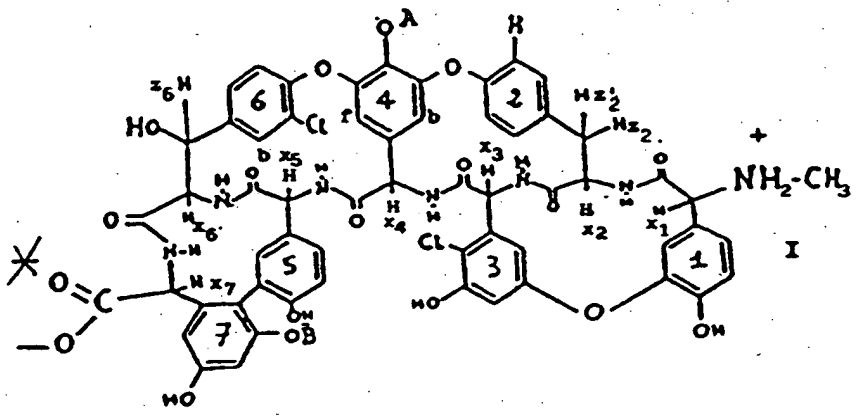
The present invention concerns a new antibiotic substance denominated antibiotic A 40926 mannosyl aglycon and the addition salts thereof, a process for preparing it from antibiotic A 40926 compl x or a factor thereof, and its use in the treatment of infectious diseases involving microorganisms susceptible to it.

Antibiotic A 40926 complex and its factors are antibiotic substances active against gram positive bacteria and Neisseriae strains, which are produced by strains of Actinomadura.

An A 40926 producing strain of Actinomadura genus has been deposited on June 8, 1984 with American Type Culture Collection (ATCC) - 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty.

Antibiotic A 40926 is a complex antimicrobial substance; five of its components have been isolated and identified as factor PA, PB, A, B and B_0.

Antibiotic A 40926 and its factors, as well as the producing microorganism and the process for their preparation, have been disclosed in European Patent Application Publication No. 177882. On the basis of the physico-chemical data and by reference to the structure of known antibiotic substances, the following formula can be attributed to the A 40926 factors (the numbering is analogous to that proposed by J. Williams in J.A.C.S., 106, 4895-4902 (1984)):



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wherein

A represents a N-(C_{11}-C_{12})acyl-aminoglucuronyl group and

B represents a mannosyl or acetylmannosyl group.

More particularly, antibiotic A 40926 factor A is the compound of the above formula wherein A represents undecanoyl-aminoglucuronyl and B represents mannosyl, antibiotic A 40926 factor B_0 is the compound of the above formula wherein A represents iso-dodecanoyl-aminoglucuronyl and B represents mannosyl.

Antibiotic A 40926 factor PA and factor PB differ from the corresponding factor A and B_0 in that the mannose unit is replaced by an acetyl-mannose unit.

Antibiotic A 40926 factors PA and PB, at least under certain fermentation conditions, are the main antibiotic products of the A 40926 producing microorganism.

Antibiotic A 40926 factors A and B are mainly transformation products of antibiotic A 40926 factor PA and factor PB respectively, and are often already present in the fermentation broth.

It has been found that antibiotic A 40926 factor PA can be transformed into antibiotic A 40926 factor A and antibiotic A 40926 factor PB can be transformed into antibiotic A 40926 factor B under basic conditions.

As a consequence, when the fermentation broth, or an antibiotic A 40926 containing extract or concentrate thereof, is allowed to stand for a certain time under basic conditions (e.g. aqueous solution of a nucleophilic base, at a pH >9 overnight,) an antibiotic A 40926 complex will be obtained which is enriched in antibiotic A 40926 factor A and factor B.

Antibiotic A 40926 mannosyl aglycon has the following characteristics (in the non-addition salt form):

A) ultraviolet absorption spectrum, which is shown in Figure 1 of the accompanying drawings, and

exhibits the following absorption maxima:

		λ max (nm)
a)	0.1 N HCl	280
b)	phosphate buffer pk 7.38	280 300(shoulder)
c)	0.1 N potassium hydroxide	298
d)	phosphate buffer pH 9.0	282 300(shoulder)

B) infrared absorption spectrum which is shown in Figure 2 of the accompanying drawings and exhibits the following absorption maxima (cm^{-1}): 3700-3100; 3000-2800 (nujol); 1655; 1620-1540; 1505; 1460 (nujol); 1375 (nujol); 1350-1250; 1210; 1150; 1020; 970; 850; 810

C) ^{1}H-NMR spectrum which is shown in Figure 3 and exhibits the following groups of signals (in ppm) at 270 MHz recorded in DMSO d_6 (hexadeuterodimethylsulfoxide) plus CF_3COOH using TMS as the internal standard (0.00 ppm), (δ = ppm; multiplicity, attributions):
2.51, s ($DMSOd_5$); 2.50, s (NCH_3); 2.88, m (Z2); 3.30, m (Z'2); 4.08, m (X6); 4.44, d (X5); 4.49, d (X7); 4.83, m (X2); 5.02, s (4F); 5.08, s (Z6); 5.31, s (anomeric proton of mannose); 5.53, d (X4); 5.78, s (4B); 6.08, d (X3); 7.70, s (6B); 6.44÷8.52 (aromatic and peptidic NH's)
d = doublet; m = multiplet; s = singlet

D) retention-time (R_t) of 1.18 relative to antibiotic L 17054 (TA3-1) (R_t = 8.78 min), when analyzed by reverse phase HPLC under the following conditions:

column: Ultraspere® ODS (5 µm) Altex (Beckman) 4.6 mm (i.d.) x 250 mm

pre-column : Brownlee Labs. RP 18 (5 µm)

eluent A: CH_3CN 10% } adjusted
(2.5 g/l) $NaH_2PO_4.H_2O$ 90% } at pH 6.0

eluent B: CH_3CN 70% } adjusted
(2.5 g/l) $NaH_2PO_4.H_2O$ 30% } at pH 6.0

elution: linear gradient from 5% to 60% of eluent B in eluent A, in 40 min

flow rate: 1.6 ml/min

U.V. detector: 254 nm

internal standard: antibiotic L 17054 (TA3-1) (Gruppo Lepetit S.p.A.)

E) R_f value of 0.39 in the following chromatographic system:

1 M NaCl containing 5 g/l of $NaH_2PO_4.H_2O$ 70
acetonitrile 30
adjusted to pH 6.0, using silanized silica gel 60 F_{254} Merck plates (layer thickness 0.25 mm)
Visualization:

- U.V. light
- Yellow color with Pauly Reagent, i.e. diazotized sulfanilic acid (J. Chromatog. 20, 171 (1965), Z. Physiol. Chem. 292, 99, (1953))
- Bioautography using B. subtilis ATCC 6633 on minimal Davis medium.

F) A fast atom bombardment (FAB) mass spectrum with $M + H^{\oplus}$ at about 1374.

On the basis of the physico-chemical characteristics and by reference to the structure of known antibiotic substances of the same class, the above formula I wherein A represents hydrogen and B represents mannosyl can be attributed to antibiotic A 40926 mannosyl aglycon.

In the present description and claims, the term "antibiotic A 40926 mannosyl aglycon" is intended to encompass the "internal salt" form as well as the possible acid and basic addition salts.

Antibiotic A 40926 mannosyl aglycon is prepared by hydrolizing antibiotic A 40926 complex enriched in factor A and factor B, antibiotic A 40926 factor A, factor B, factor B_0 or mixtures thereof under controlled acidic conditions.

These controlled acid conditions are represented by a concentrated aqueous solution of a mineral or organic strong acid optionally in the presence of an aprotic organic solvent. Preferred examples of strong mineral acids are sulfuric and phosphoric acid.

A preferred strong organic acid is trifluoroacetic acid.

Preferred aprotic organic solvents are alicyclic or cyclic alkyl ethers such as dioxane and tetrahydrofuran, lower alkyl sulfoxides such as dimethylsulfoxide and lower alkyl amides such as dimethylformamide.

The reaction temperature is generally kept between 0 °C and the reflux temperature of the reaction mixture. In many instances it is between 15 °C and 75 °C, while a preferred temperature is between 20 °C and 55 °C and most preferably it is room temperature.

The reaction time varies depending on the specific reaction parameters and since the reaction course may be followed by TLC or HPLC techniques, the man skilled in the art is capable of monitoring the reaction course and deciding when the reaction may be considered complete.

A preferred embodiment of the process of the invention is represented by the controlled hydrolysis of antibiotic A 40926 complex or a pure factor thereof to give antibiotic A 40926 mannosyl aglycon in the presence of aqueous 80-95% trifluoroacetic acid at room temperature.

Another preferred embodiment of the process of the invention is represented by the controlled hydrolysis of antibiotic A 40926 mannosyl aglycon in the presence of a mixture 2:1 to 1:2 of aqueous 1-2 N sulfuric acid and dioxane.

The purification of crude antibiotic A 40926 mannosyl aglycon as recovered at the end of the hydrolysis step may be accomplished according to known per se techniques, such as precipitation by addition of non-solvents, extraction with solvents and chromatography.

Preferred chromatographic procedures include column chromatography and the most preferred chromatographic procedure is reverse-phase column chromatography.

A suitable reverse-phase liquid chromatography procedure, preferably employs stainless steel columns under moderate pressure (5-50 bar) or at high pressure (100-200 bar). The solid phase may be a silanized silica gel with a hydrocarbon phase at (2-18) carbon atoms (most preferably C 18) or phenyl group, and the eluent is a mixture of a polar water-miscible solvent and an aqueous buffer at a pH compatible with the resin (preferably pH 3-8).

Representative examples of polar water-miscible solvents are: water-soluble alcohols, (such as methanol, ethanol, iso-propanol, n-butanol), acetone, acetonitrile, lower alkyl alkanoates (such as ethyl acetate), tetrahydrofuran, dioxane and dimethylformamide and mixtures thereof; the preferred polar water-miscible solvent being acetonitrile.

The eluted fractions are assayed for their antibiotic content by means of the usual bioassays, such as paper-disc or agar-diffusion assays, on susceptible microorganisms. Examples of susceptible organisms are Bacillus subtilis and S. aureus.

The purification as well as the reaction are also conveniently monitored by TLC or HPLC techniques.

A preferr d HPLC technique is represented by a reverse-phase HPLC using a column of porous and spheric particles of silanized silica gel functionalized with C-18 alkyl groups having a diameter preferably of 5 micrometers (such as 5 µm Ultrasphere ® ODS Altex; Beckman Co.), a pre-column which is a silica gel functionalized with C-18 alkyl groups (such as RP 18 Brownlee Labs) and an eluent which is a linear

gradient mixture of a polar water miscible solvent, such as one of those described above, in an aqueous buffered solution.

Preferably this solution is adjusted to pH 5-7. A most preferred eluent is represented by a linear gradient from 5 to 60% of eluent B in elu nt A wherein luent A is a mixture of acetonitrile/aqueous buffer, pH 5-7, 10:90 and eluent B is a mixture of acetonitrile/aqueous buffer, pH 5-7, 70:30. As known in the art, many substances can be used as internal standards. A very convenient one is, in this case, antibiotic L 17054 which has a retention time close to the compounds of the invention in this HPLC system. This standard substance is known and has been described in European Patent Application Publication No. 0119575.

The antibacterial activity of the compound of the invention can be demonstrated in vitro by means of standard dilution tests on different microorganism cultures.

Culture media and growth conditions for MIC (minimal inhibitory concentration) determinations were as follows: Isosensitest® broth (Oxoid), 24 h, for staphylococci, Strep. faecalis and Gram-negative bacteria (Escherichia coli); Todd-Hewitt broth (Difco), 24 h for other streptococcal species; GC base broth (Difco) + 1% Isovitalex® (BBL), 48 h, CO_2-enriched atmosphere for Neisseria gonorrhoeae; GB base agar (Difco) + 1% Isovitalex + 0.001% hemin, 24 h for Haemophilus influenzae; AC broth (Difco), 24 h, anaerobic atmosphere for Clostridium perfringens; Wilkins-Chalgren agar (ref: T.D. Wilkins & S. Chalgren, 1976, Antimicrob. Ag. Chemother. 10, 926), 48 h, anaerobic atmosphere for the other anaerobes (C. difficile, Propionibacterium acnes, Bacteroides fragilis); PPLO broth (Difco) + 10% horse serum + 1% glucose, 48 h for Mycoplasma gallisepticum. Incubation was at 37° C. Inocula were as follows: 1% (v/v) of a 48 h broth culture for M. gallisepticum; about 10^4-10^5 colony-forming units/ml for other broth dilution MICs; about 10^4-10^5 bacteria/spot (inoculated with a multipoint inoculator) for agar dilution MICs (H. influenzae, C. difficile, P. acnes, B. fragilis).

The minimal inhibitory concentrations (MIC, µg/ml) on some microorganisms are reported below in Table I.

TABLE I

Strain	Antibiotic A 40926 mannosyl aglycon M.I.C. (µg/ml)
Staph. aureus Tour	0.25
Staph. aureus Tour (L165) 10^6 cfu/ml	1
Staph aureus Tour 30% bovine serum	2
Staph. epidermidis ATCC 12228	0.5
Strep. pyogenes C203	1
Strep. pneumoniae UC41	1
Strep. faecalis ATCC 7080	1
Strept. mitis clin. isolate	1
Clostridium perfringens ISS30543	0.13
Clostridium difficile ATCC 9689	2
Bacteroides fragilis ATCC 23745	64
Propionibacterium acnes ATCC 6919	1
Neisseria gonorrhoeae ISM68/126	32
Haemophilus influenzae type b ATCC 19418	64
Escherichia coli SKF 12140	>128
Mycoplasma gallisepticum S6 Weybridge	>128

The antimicrobial activity of the compound of the invention is confirmed also in in vivo experiments conducted essentially as described by R. Pallanza et al., J. Antimicrob. Ch mother. 11, 419 (1983).

Th xperimental infection was induced in mice by intrap riton ally administ ring a suspension of S. pyogenes C 203. Inocula had been adjusted so that the untreated animals di d of septic mia within 48 h. Animals were treated subcutaneously with the test compound about 30 min aft r infection.

The ED_{50} value was calculated on the 10th day by the method of Spearman and Karber (D.J. Finney "Statistical M thods in Biological Assay", Griffin, page 524, 1952) on the basis of the percentage of survival at each dose.

In the above conditions the ED_{50} for antibiotic A 40926 mannosyl aglycon was 3.8 mg/kg.

Antibiotic A 40926 mannosyl aglycon possesses acid and basic functions and can form salts according to conventional procedures.

Representative and suitable acid addition salts of the compounds of the invention include those salts formed by standard reaction with both organic and inorganic acids such as, for example, hydrochloric, hydrobromic, sulfuric, phosphoric, acetic, trifluoroacetic, trichloroacetic, succinic, citric, ascorbic, lactic, maleic, fumaric, palmitic, cholic, pamoic, mucic, glutamic, camphoric, glutaric, glycolic, phthalic, tartaric, lauric, stearic, salicylic, methanesulfonic, benzenesulfonic, sorbic, picric, benzoic and cinnamic acids.

Representative examples of these bases are: alkali metal or alkaline-earth metal hydroxide such sodium, potassium, and calcium, hydroxide; ammonia and organic amines aliphatic, alicyclic or aromatic such as methylamine, dimethylamine, trimethylamine, and picoline.

The transformation of the free amino or non-salt compounds of the invention into the corresponding addition salts, and the reverse, i.e. the transformation of an addition salt of a compound of the invention into the non-salt or free amino form, are within the ordinary technical skill and are encompassed by the present invention.

For instance antibiotic A 40926 mannosyl aglycon can be transformed into the corresponding acid or base addition-salt by dissolving the non-salt form in an aqueous solvent and adding a slight molar excess of the selected acid or base. The resulting solution or suspension is then lyophilized to recover the desired salt.

In case the final salt is unsoluble in a solvent where the non-salt form is soluble it is recovered by filtration from the organic solution of the non-salt form after addition of the stoichiometric amount or a slight molar excess of the selected acid or base. The non-salt form can be prepared from a corresponding acid or base salt dissolved in an aqueous solvent which is then neutralized to free the non-salt form.

When following the neutralization desalting is necessary, a common desalting procedure may be employed. For example, column chromatography on silanized silica gel, non-functionalized polystyrene, acrylic and controlled pore polydextrane resins (such as Sephadex LH 20) or activated carbon may be conveniently used. After eluting the undesired salts with an aqueous solution, the desired product is eluted by means of a linear gradient or a step-gradient of a mixture of water and a polar or apolar organic solvent, such as acetonitrile/water from 50:50 to about 100% acetonitrile.

As it is known in the art, the salt formation either with pharmaceutically acceptable acids (bases) or non-pharmaceutically acceptable acids (bases) may be used as a convenient purification technique. After formation and isolation, the salt form of an A 40926 antibiotic can be transformed into the corresponding non-salt or into a pharmaceutically acceptable salt.

In some instances, the base addition salt of antibiotic A 40926 mannosyl aglycon is more soluble in water and hydrophilic solvents.

Antibiotic A 40926 mannosyl aglycon is active against gram-positive bacteria which are responsible for many widely diffused infections. Because of the increasing resistance of these pathogens to the usual therapeutic treatments, the need for new antibiotic substances is still great.

In general for antibacterial treatment antibiotic A 40926 mannosyl aglycon as well as the non-toxic pharmaceutically acceptable salts thereof or mixture thereof, can be administered by different routes such as topically or parenterally. The parenteral administration is, in general, the preferred route of administration.

Compositions for injection may take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain adjuvants such as suspending, stabilizing and/or dispersing agents.

Alternatively, the active ingredient may be in powder form for reconstitution at the time of delivery when a suitable vehicle, such as sterile water, is added thereto.

Depending on the route of administration, these compounds can be formulated into various dosage forms.

In some instances, it may be possible to formulate the compounds of the invention in enteric-coated dosage forms for oral administration which may be prepared as known in the art (see for instance "Remington's Pharmaceutical Sciences", fifteenth edition, Mack Publishing Company, Easton, Pennsylvania, USA, page 1614).

This could be especially the case when the absorption of the antimicrobial substance in the enteric tract is particularly desired while passing unaltered through the gastric tract.

The amount of active principle to be administered depends on various factors such as the size and condition of the subject to be treated, the route and frequency of administration, and the causative agent

involv d.

The antibiotic substances of the present invention, namely antibiotic A 40926 mannosyl aglycon and the physiologically acceptable salts thereof, are generally effective at a daily dosage of between about 0.5 and 50 mg of active ingredient per kilogram of pati nt body weight, optionally divid d into 1 to 4 administrations per day.

Particularly desirable compositions are those prepared in dosage units containing from about 100 to about 5,000 mg per unit.

Sustained-action formulations can be prepared based on different mechanisms and methods, as known in the art.

A preferred method for preparing a sustained-action formulation containing antibiotic A 40926 mannosyl aglycon, involves the use of a water insoluble form of this antibiotic suspended in an aqueous or oily medium.

Preparation of pharmaceutical compositions:

A unit dosage form for intramuscular injection is prepared with 5 ml of sterile suspension USP containing 8% propylene glycol and 1,000 mg of antibiotic A 40926 mannosyl aglycon.

A unit dosage form for intramuscular injection is prepared with 5 ml of sterile water USP containing 500 mg of antibiotic A 40926 mannosyl aglycon.

A unit dosage form for intramuscular injection is prepared with 2,000 mg of antibiotic A 40926 mannosyl aglycon sodium salt suspended in 5 ml of sterile water for injection.

Furthermore, the antibiotic substance of the invention is useful for suppressing the growth of Clostridium difficile which causes pseudomembranous colitis in the intestine. The antibiotic could be used in the treatment of pseudomembranous colitis by the oral administration of an effective dose of the antibiotic or a pharmaceutically-acceptable salt thereof, prepared in a pharmaceutically-acceptable dosage form. For such use, the antibiotic can be administered in gelatin capsules or in liquid suspension.

Besides their activity as medicaments, antibiotic A 40926 mannosyl aglycon and the pharmaceutically acceptable salts thereof, can be used as animal growth promoters.

For this purpose, a compound of the invention is administered orally in a suitable feed. The exact concentration employed is that which is required to provide for the active agent in a growth promotant effective amount when normal amounts of feed are consumed.

The addition of the active compound of the invention to animal feed is preferably accomplished by preparing an appropriate feed premix containing the active compound in an effective amount and incorporating the premix into the complete ration.

Alternatively, an intermediate concentrate or feed supplement containing the active ingredient can be blended into the feed.

The way in which such feed premixes and complete rations can be prepared and administered are described in reference books (such as "Applied Animal Nutrition", W.H. Freedman and CO., S. Francisco, USA, 1969 or "Livestock Feeds and Feeding" O and B books, Corvallis, Oregon, USA, 1977) and are incorporated herein by reference.

The following Examples further illustrate the invention and, as such, should not be construed as limiting its scope.

Example 1

a) A sample (214 mg) of antibiotic A 40926 complex AB (see Preparation 3, below) is dissolved in a trifluoroacetic acid/water 9/1 (v/v) mixture (30 ml). After 48 h at room temperature the mixture is evaporated under vacuum at room temperature to 1/10 of the original volume, rinsed with 30 ml of water and then extracted twice with ethyl acetate. The aqueous phase contained mainly the A 40926 mannosyl aglycon and trifluoroacetic acid.

b) This aqueous phase is neutralized with 1 N sodium hydroxide and then loaded on a column (bed height 5 cm) of silanized silica gel 60, 70-230 mesh (50 ml; Merck Co. art. 7719) in water. The column is washed with distilled water and then eluted with acetonitrile-water 1/1 (v/v).

Fractions of 20 ml are collected, analyzed by HPLC (or TLC) and thos containing antibiotic A 40926 mannosyl aglycon are pooled, concentrated to a small volume by azeotropic distillation with n-butanol and th n freeze-dried to give 124 mg of antibiotic A 40926 mannosyl aglycon.

Example 2

The same procedure of example 1 is repeated but the starting material is reacted with the aqueous trifluoroacetic acid for 2 h at about 65° C instead of at room t mperature overnight. Yield 110 mg of final product.

Example 3

The same procedure of example 1 is repeated but using a mixture of 2 N aqueous sulfuric acid and dioxane (one to one ratio) instead of aqueous trifluoroacetic acid. The reaction temperature is, in this case, about 65° C, overnight. The yields are similar to those obtained in the foregoing examples.

Example 4

The procedure of example 1 is repeated using
a) antibiotic A 40926 factor A
b) antibiotic A 40926 factor B
as the starting materials instead of antibiotic A 40926 complex. The yields are similar to those obtained in the foregoing examples.

Example 5

Antibiotic A 40926 mannosyl aglycon as obtained according to the procedure of example 1a is further purified as follows:
the aqueous phase is neutralized with 1 N aqueous sodium hydroxide and then concentrated to about 18 ml under vacuum.

The obtained solution is applied in two subsequent chromatographic runs to a stainless steel column (2 cm diameter) packed with 20 g of 10 micron RP 18 silica gel (Lichrosorb® RP18 Merck Co., art. 9334) equilibrated with a solvent consisting of acetonitrile: 18 mM sodium phosphate buffer pH 6.0, 17:83 (v/v).

The column is part of a Chromatospac Modulprep® unit. (Joben Yvon, 16-18 Rue du Canal 91160, Longjnneau, France). The column is eluted with acetonitrile: 18 mM sodium phosphate buffer pH 6.0, 17:83 (v/v). The eluted fractions are monitored at 275 nm using a UVICORD S UV monitor (LKB Co.) and are analyzed by HPLC and TLC.

The fractions of the two chromatographic runs containing antibiotic A 40926 mannosyl aglycon are pooled and concentrated under vacuum to remove the organic solvent. The resulting aqueous solution is then chromatographed and worked up as described in example 1b giving 66 mg of A 40926 mannosyl aglycon lyophilizate.

Preparation of the starting materials:

Preparation 1:

Fermentation of Actinomadura sp. ATCC 39727

A culture of antibiotic A 40926 producing strain (Actinomadura sp. ATCC 39727) is grown on oatmeal agar slant for 2-3 weeks at 28° C and used to inoculate a 500 ml Erlenmeyer flask containing 100 ml of medium composed of 0.5% meat extract, 0.5% autolyzed yeast, 0.5% peptone, 0.3% casein hydrolyzed, 2% glucose, 0.15% NaCl (pH 7.5 before sterilization).

The flask is incubated at 28° C on a rotary shaker at 200 rpm for about 72 h and then the culture is transferred to a fermentor containing 4 l of the above medium. This culture is grown at 28° C for about 72 h with air-flow of about two liters per minute and stirring at about 900 rpm. Then, it is used to inoculate a 200 l fermentor of the same medium. This fermentor is aerated with 100 l per minute of sterile air and is stirred at 250 rpm at about 28° C. The antibiotic production is monitored by the paper-disc agar diffusion method using B. subtilis on a minimal medium as the test organism. The maximum activity is obtained after 72-96 h.

Preparation 2:

Recovery of antibiotic A 40926

A) The fermentation broth is cooled to 4° C, brought to pH 9.5 and stirred. After about 1 h it is filtered and the filtrate is adjusted to pH about 3.5 with an aqueous mineral acid. The mixture is stirred for 30 min at 4° C and then filtered with (Hyflo-FloMa ®) filter aid. The clear filtrate is discharged and the filter cake is suspended in deionized water, adjusted to pH about 8.5, stirred and then filtered. The recovered cake is subjected to the same procedure. The pooled filtrates contain antibiotic A 40926.

B) Swollen D-Ala-D-Ala-ε-aminocaproyl-Sepharose® modified matrix (2 l) is added to the fermentation broth obtained according to Preparation 1 (after filtering it and bringing the pH of the clear filtrate to about 8.5) or to the pooled filtrate obtained according to the above Preparation 2 A. After stirring overnight at room temperature, the resin is recovered by filtration and is washed sequentially with about 2 x 10 l of 0.45 mM HCl-TRIS buffer pH 7.5 (TRIS = 2-amino-2-hydroxymethyl-1,3-propanediol) which contains 5% (w/v) NaCl and then with distilled water (4 x 20 l). The A 40926 antibiotic is eluted from the resin with 1% (w/v) ammonia hydrate (2 x 20 l). The eluates are left overnight at room temperature and then concentrated to a small volume (about 2.5 l). Water is eliminated by azeotropical distillation with n-butanol. Petroleum ether is then added, precipitating 3.4 g of crude antibiotic A 40926 complex.

Preparation 3:

Purification of antibiotic A 40926 complex AB

Crude antibiotic A 40926 complex obtained essentially following the procedure of the above Preparation 2, (750 mg; HPLC titre 70%) is dissolved in 400 ml of water, adjusted to pH 7.5 and filtered. The filtrate is then subjected to affinity chromatography on a D-Ala-D-Ala-ε-aminocaproyl-Sepharose column (50 ml of swollen resin; bed height = 5 cm). The column, equilibrated with 0.16% (w/v) ammonia containing 2 M NaCl adjusted to pH 7.5 with HCl, is developed sequentially with the following three buffer solutions:

- buffer A: 0.16% (w/v) ammonia contaning 2M NaCl adjusted to pH 7.5 with HCl, (2.6 column bed volumes);
- buffer B: 0.16% (w/v) ammonia containing 2M NaCl adjusted to pH 9.5 with HCl (16 column bed volumes);
- buffer C: 1% (w/v) aqueous ammonia pH 11.4 (2.6 column bed volumes).

Buffer C elutes antibiotic A 40926 complex AB in a single fraction. This eluted fraction is adjusted to pH 7.0 and reapplied to the same affinity column buffered with 10 mM TRIS-HCl pH 7.0. The column is washed with distilled water until desalting is complete. The antibiotic is then eluted with 2 column bed volumes of 0.39% (w/v) aqueous ammonia pH 11.0.

The eluted fractions are concentrated to a small aqueous mixture and then freeze-dried. Pure antibiotic A 40926 complex AB (374 mg) is obtained.

Preparation 4:

Isolation of antibiotic A 40926 factors A and B

A) Antibiotic A 40926 complex as obtained according to Preparation 2 (3.3 g) or antibiotic A 40926 complex AB as obtained according to Preparation 3 (2.3 g) is suspended in 0.5 l of water, stirred and then filtered. The clear filtrate is applied to a silanized silica gel column (200 g; bed h 18 cm; silanized Silicagel 60; 70-230 mesh, Merck Inc.) pre-equilibrated with solution A (0.001 M aqueous sodium EDTA containing 0.25% (w/v) $NaH_2PO_4.H_2O$ and 2.5% (w/v) NaCl adjusted to pH 6.0 with NaOH). The column is eluted with a linear gradient from 0% to 40% (v/v) of acetonitrile in solution A with a total volume of about 7 l in about 48 h. Fractions of about 15.5 ml are collected and assayed by bioassay on Bacillus subtilis and analyzed by HPLC. Fractions having a similar antibiotic content are pooled. Fractions No. 310-330 and No. 348-365 contained the antibiotic substances denominated, respectively, A 40926 factor A and A 40926 factor B.

B) The pooled fractions containing the single A 40926 factors A and B are concentrated under reduced pressure to remove acetonitrile, diluted with water (about twice the volume of the initial solutions) and applied to a silanized silica gel column of the type described above (volume of the swollen matrix: 50 ml; bed height of 15 cm). The column is washed with deionized water until desalting is complete and finally developed with acetonitrile/wat r 60:40 (v/v).

The eluted fractions ar concentrated under reduced pressure and the residues ar freez -dried to obtain 134 mg of antibiotic A 40926 factor A from the first group of eluted fractions (fractions 310-330 above) and 206 mg of A 40926 factor B from the second group of eluted fractions (fractions 348-365,

above).

Preparation 5:

Isolation of antibiotic A 40926 factor PA and factor PB

By essentially following the procedure of Preparation 2A and the first steps of the procedure of Preparation 2B, the antibiotic linked to the resin is eluted with 1% (w/v) ammonia hydrate (2 x 20 l). The eluates are adjusted to pH 7.8 with sulfuric acid and concentrated to a small volume under vacuum by azeotropical distillation with n-butanol to obtain an aqueous concentrate which is then filtered on paper. The recovered filtrate contains antibiotic A 40926 factor PA, A 40926 factor PB and minor amounts of A 40926 factor A and factor B (HPLC).

A sample (10 ml) of this aqueous concentrate containing about 50 mg/ml of pure antibiotic A 40926 complex (HPLC analysis) is filtered on 5 micrometer pore-size filter (Acrodisc®; Gelman Science Inc.) and then applied to a stainless steel column (diameter = 2 cm) containing 20 g of an octadecyl silyl reverse-phase silica gel (Lichrisorb RP 18, Merck Inc.; particle size 10 µm). The silica gel is then packed under moderate pressure (nominal pressure about 14 bar) in a stainless steel column of a Chromatospac Modulprep apparatus (Joben Yvon, France) and equilibrated with a mixture consisting of acetonitrile and 18 mM sodium phosphate buffer pH 6.0, 25:75 (v/v). The elution is carried out using the same solvent mixture used for the equilibration at a flow rate of about 10.5 ml/min. The eluate is monitored by bioassay on Bacillus subtilis and by HPLC.

Those fractions having similar antibiotic content are pooled and the homogeneous fractions of 5 chromatographic runs are concentrated to evaporate the organic solvent.

The resulting solution is diluted with aqueous 1M sodium chloride to twice the original volume and is applied to a silanized silica gel column (50 g; bed height 5 cm; Silanized silica gel 60; Merck Inc.) equilibrated with water.

The column is washed with deionized water until desalting is complete (no AgCl precipitation in the eluates after addition of aqueous $AgNO_3$) and then eluted with acetonitrile:water 1:1 (v/v). The eluates having similar antibiotic content (HPLC analysis) are pooled, concentrated to a small volume by azeotropical distillation with n-butanol to obtain an aqueous phase which is then freeze-dried. Yields:

antibiotic A 40926 factor PA : 55 mg
antibiotic A 40926 factor PB : 51 mg
antibiotic A 40926 factor A : 38 mg
antibiotic A 40926 factor B_0 : 33 mg

Preparation 6:

Transformation of antibiotic A 40926 factor PA and antibiotic A 40926 factor PB into antibiotic A 40926 factor A and factor B, respectively

Antibiotic A 40926 factor PA and antibiotic A 40926 factor PB (50 mg) are separately dissolved in 2.5 ml of aqueous 1% (w/v) NH_4OH and the resulting solutions are kept for about 24 h at room temperature with stirring.

Antibiotic A 40926 factor A is obtained from the solution originally containing antibiotic A 40926 factor PA, and antibiotic A 40926 factor B is obtained from the solution originally containing antibiotic A 40926 factor PB by removing water by azeotropic distillation with n-butanol, precipitating with ethyl ether and collecting the precipitate by filtration (yield about 75%).

Preparation of D-Ala-D-Ala-Sepharose

Activated CH-Sepharose 4B (Pharmacia Fine Chemicals) (1g) is swollen for 15 minutes in 1 mM cold ice hydrochloric acid and washed with the same solution.

The obtained gel (about 3 ml) is mixed with a solution of D-alanyl-D-alanine (30 mg) in 0.5 M sodium chloride and 0.1 M sodium bicarbonate buff r at pH 8.

The mixture is rotat d end-over-end for 1 hour at room temperature.

After the coupling r action is completed, the ligand excess is wash d off with the buffer. The unlinked activated groups of the dextrane support are blocked by treating them with 1 M ethanolamine hydrochloride at pH 9 for 1 hour.

Then the Sephadex-ε-aminocaproyl-D-alanyl-D-alanine modified matrix is recovered by filtration and thoroughly washed alternativ ly with 0.5 M sodium chloride and 0.1 M sodium acetate pH 4, and with 0.5 M sodium chloride and 0.1 M tris(hydroxymethyl)aminomethane buffer pH 8. (four times).

Claims

Claims for the following Contracting States : BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. Antibiotic A 40926 mannosyl aglycon and the addition salts thereof which has the following characteristics, in the non-addition salt form:

A) ultraviolet absorption spectrum which exhibits the following absorption maxima:

		λ max (nm)
a)	0.1 N HCl	280
b)	phosphate buffer pH 7.38	280 300 (shoulder)
c)	0.1 N potassium hydroxide	298
d)	phosphate buffer pH 9.0	282 300 (shoulder)

B) infrared absorption spectrum which exhibits the following absorption maxima (cm^{-1}):
3700-3100; 3000-2800 (nujol); 1655; 1620-1540; 1505; 1460 (nujol); 1375 (nujol); 1350-1250; 1210; 1150; 1020; 970; 850; 810

C) ^{1}H-NMR spectrum which exhibits the following groups of signals (in ppm) at 270 MHz recorded in DMSO d_6 (hexadeuterodimethylsulfoxide) plus CF_3COOH using TMS as the internal standard (0.00 ppm), (δ = ppm, multiplicity, attributions):
2.51, s ($DMSOd_5$); 2.50, s (NCH_3); 2.88, m (Z2); 3.30, m (Z'2); 4.08, m (X6); 4.44, d [X5]; 4.49, d (X7); 4.83, m (X2); 5.02, s (4F); 5.08, s (Z6); 5.31, s (anomeric proton of mannose); 5.53, d (X4); 5.78, s (4B); 6.08, d (X3); 7.70, s (6B); 6.44÷8.52 (aromatic and peptidic NH's)

D) retention-time (R_t) of 1.18 relative to antibiotic L 17054 (TA3-1) (R_t = 8.78 min), when analyzed by reverse phase HPLC under the following conditions:

column: Ultraspere® ODS (5 µm) Altex (Beckman)
4.6 mm (i.d.) x 250 mm

pre-column : Brownlee Labs. RP 18 (5 µm)

eluent A: CH_3CN 10% } adjusted
(2.5 g/l) $NaH_2PO_4.H_2O$ 90% } at pH 6.0

eluent B: CH_3CN 70% } adjusted
(2.5 g/l) $NaH_2PO_4.H_2O$ 30% } at pH 6.0

elution: linear gradient from 5% to 60% of eluent B in eluent A, in 40 min

flow rate: 1.6 ml/min

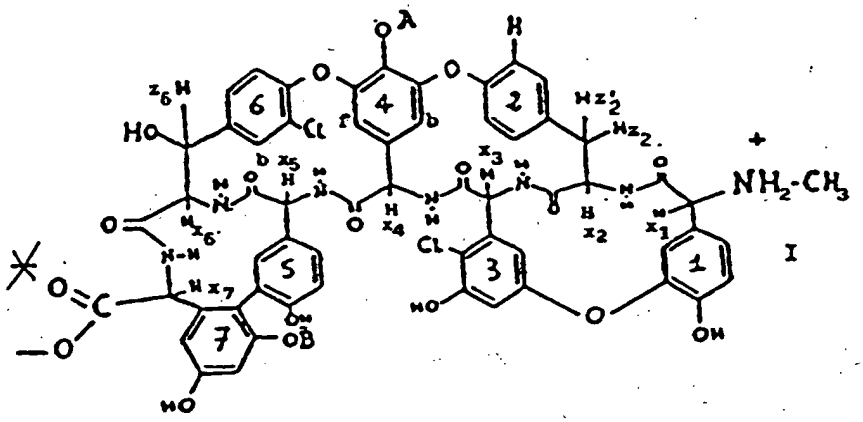
U.V. detector: 254 nm

internal standard: antibiotic L 17054 (TA3-1)
(Gruppo Lepetit S.p.A.)

E) R_f value of 0.39 in the following chromatographic system:
1 M NaCl containing 5 g/l of $NaH_2PO_4.H_2O$ 70
acetonitrile 30
adjusted to pH 6.0, using silanized silica gel 60 F_{254} Merck plates (layer thickness 0.25 mm)
Visualization:
- U.V. light
- Yellow color with Pauly Reagent, i.e. diazotized sulfanilic acid (J. Chromatog. 20, 171 (1965), Z. Physiol. Chem. 292, 99, (1953))
- Bioautography using B. subtilis ATCC 6633 on minimal Davis medium.

F) A fast atom bombardment (FAB) mass spectrum with M + H $^{\bullet}$ at about 1374.

2. Antibiotic A 40926 mannosyl aglycon and the addition salts thereof, which have the following formula, in the non-addition salt form:



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wherein

A represents hydrogen, and

B represents mannosyl.

3. A process for preparing a compound of claim 1 or 2 which comprises hydrolyzing antibiotic A 40926 complex enriched in factors A and B, antibiotic A 40926 factor A, antibiotic A 40926 factor B or B_0 or mixtures thereof in a concentrated aqueous solution of a mineral or organic strong acid optionally in the presence of an aprotic organic solvent.

4. A process as claimed in claim 3 wherein the mineral acid is sulfuric or phosphoric acid

5. A process as claimed in claim 3 wherein the organic acid is trifluoroacetic acid.

6. A process as claimed in claim 3 wherein the aprotic organic solvents are alicyclic or cyclic alkyl ethers, lower alkyl sulfoxides or lower alkyl amides.

7. A process as claimed in 6 wherein the aprotic organic solvent is dioxane, tetrahydrofuran, dimethysulfoxide or dimethylformamide.

8. A compound of claim 1 or 2 for use as a medicine.

9. Use of a compound of claim 1 or 2 for the preparation of a medicament for antimicrobial use.

10. A pharmaceutical composition comprising a compound of claim 1 or 2 in admixture with a pharmaceutically acceptable carrier.

Claims for the following Contracting States : AT, GR, ES

1. A process for preparing antibiotic A 40926 mannosyl aglycon and the addition salts thereof which has the following characteristics, in the non-addition salt form:

A) ultraviolet absorption spectrum which exhibits the following absorption maxima:

		λ max (nm)
a)	0.1 N HCl	280
b)	phosphate buffer pH 7.38	280 300 (shoulder)
c)	0.1 N potassium hydroxide	298
d)	phosphate buffer pH 9.0	282 300 (shoulder)

B) infrared absorption spectrum which exhibits the following absorption maxima (cm^{-1}):
3700-3100; 3000-2800 (nujol); 1655; 1620-1540; 1505; 1460 (nujol); 1375 (nujol); 1350-1250; 1210; 1150; 1020; 970; 850; 810

C) ^{1}H-NMR spectrum which exhibits the following groups of signals (in ppm) at 270 MHz recorded in DMSO d_6 (hexadeuterodimethylsulfoxide) plus CF_3COOH using TMS as the internal standard (0.00 ppm), (δ = ppm, multiplicity, attributions):
2.51, s ($DMSOd_5$); 2.50, s (NCH_3); 2.88, m (Z2); 3.30, m (Z'2); 4.08, m (X6); 4.44, d (X5); 4.49, d (X7); 4.83, m (X2); 5.02, s (4F); 5.08, s (Z6); 5.31, s (anomeric proton of mannose); 5.53, d (X4); 5.78, s (4B); 6.08, d (X3); 7.70, s (6B); 6.44÷8.52 (aromatic and peptidic NH's)

D) retention-time (R_t) of 1.18 relative to antibiotic L 17054 (TA3-1) (R_t = 8.78 min), when analyzed by reverse phase HPLC under the following conditions:

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(2.5 g/l) $NaH_2PO_4.H_2O$ 30% } at pH 6.0

elution: linear gradient from 5% to 60% of eluent B in eluent A, in 40 min

flow rate: 1.6 ml/min

U.V. detector: 254 nm

internal standard: antibiotic L 17054 (TA3-1) (Gruppo Lepetit S.p.A.)

E) R_f value of 0.39 in the following chromatographic system: 1 M NaCl containing 5 g/l of $NaH_2PO_4.H_2O$ 70
acetonitrile 30
adjust d to pH 6.0, using silanized silica gel 60 F_{254} Merck plates (layer thickness 0.25 mm)

Visualization:
- U.V. light
- Yellow color with Pauly Reagent, i.e. diazotized sulfanilic acid (J. Chromatog. 20, 171 (1965), Z. Physiol. Chem. 292, 99, (1953))
- Bioautography using B. subtilis ATCC 6633 on minimal Davis medium.

F) A fast atom bombardment (FAB) mass spectrum with M + H $^{\oplus}$ at about 1374, which comprises hydrolyzing antibiotic A 40926 complex enriched in factors A and B, antibiotic A 40926 factor A, factor B_0 or factor B_0 or mixtures thereof in a concentrated aqueous solution of a mineral or organic strong acid optionally in the presence of an aprotic organic solvent.

2. A process as claimed in claim 1 wherein the mineral acid is sulfuric or phosphoric acid.

3. A process as claimed in claim 1 wherein the organic acid is trifluoroacetic acid.

4. A process as claimed in claim 1 wherein the aprotic organic solvents are alicyclic or cyclic alkyl ethers, lower alkyl sulfoxides or lower alkyl amides.

5. A process as claimed in claim 4 wherein the aprotic organic solvent is dioxane, tetrahydrofuran, dimethylsulfoxide or dimethylformamide.

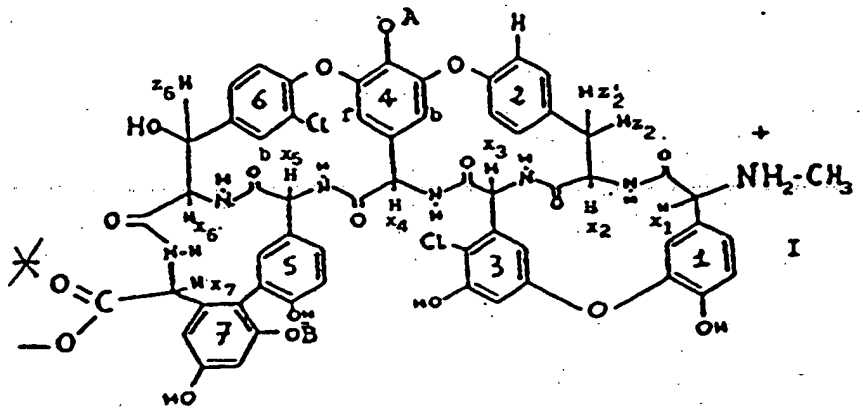
6. A process as claimed in claim 1 wherein the reaction temperature is between 0 °C and the reflux temperature.

7. A Process as claimed in claim 6 wherein the reaction temperature is between 15 °C and 75 °C.

8. A process as claimed in claim 1 and 6 wherein the strong acid is 85-95% aqueous trifluoroacetic acid and the reaction temperature is room temperature.

9. A process as claimed in claim 1 wherein the hydrolysis is carried out in the presence of a mixture 2:1 to 1:2 of aqueous 1-2 N sulfuric acid and dioxane.

10. A process according to claim 1 for preparing antibiotic A 40926 mannosyl aglycon and the addition salts thereof, which has the following formula, in the non-addition salt form:



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wherein
- A represents hydrogen, and
- B represents mannosyl.

Revendications

Revendications pour les Etats contractants suivants : BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. Mannosyl aglycone de l'antibiotique A 40926 et ses sels d'addition, qui présente les caractéristiqu s suivantes sous la forme non-sel d'addition :

A) spectre d'absorption ultraviolet présentant les maxima d'absorption suivants :

		λ max (nm)
a)	HCl 0,1 N	280
b)	tampon phosphate pH 7,38	280 300 (épaulement)
c)	hydroxyde de potassium 0,1 N	298
d)	tampon phosphate pH 9,0	282 300 (épaulement)

B) spectre d'absorption IR présentant les maxima d'absorption suivants (cm^{-1}) :
3700-3100 ; 3000-2800 (nujol) ; 1655 ; 1620-1540 ; 1505 ; 1460 (nujol) ; 1375 (nujol); 1350-1250 ; 1210 ; 1150 ; 1020; 970 ; 850 ; 810

C) spectre de RMN-^{1}H présentant les groupes de signaux suivants (en ppm) à 270 MHZ, enregistrés dans du DMSO d_6 (sulfoxyde d'hexadeutérodiméthyle) plus CF_3 COOH en utilisant le TMS comme étalon interne (0,00 ppm), (δ = ppm, multiplicité, attributions) : 2,51, s ($DMSOd_5$) ; 2,50, s (NCH_3) ; 2,88, m (Z2) ; 3,30, m
(Z'2) ; 4,08, m (X6) ; 4,44, d (X5) ; 4,49, d (X7) ; 4,83, m (X2) ; 5,02, s (4F) ; 5,08, s (Z6) ; 5,31, s (proton anomère du mannose) ; 5,53, d (X4) ; 5,78, s (4B) ; 6,08, d (X3) ; 7,70, s (6B) ; 6,44+8,52 (NH aromatiques et peptidiques)

D) temps de rétention (R_t) de 1,18 par rapport à l'antibiotique L 17054 (TA3-1) (R_t = 8,78 min), lorsqu'il est analysé par HPLC en phase inversée dans les conditions suivantes :

Colonne : Ultrasphere® ODS (5 µm) Altex (Beckman)
4,6 mm (d.i.) x 250 mm

Pré-colonne : Brownlee Labs. RP 18 (5 µm)

Eluant A : CH_3CN 10 % } ajusté à
(2,5 g/l) $NaH_2PO_4.H_2O$ 90 % } pH 6,0

Eluant B : CH_3CN 70 % } ajusté à
(2,5 g/l) $NaH_2PO_4.H_2O$ 30 % } pH 6,0

Elution : gradient linéaire de 5 % à 60 % d'éluant B dans l'éluant A, en 40 min.

Débit : 1,6 ml/min

Détecteur UV : 254 nm

Etalon interne : antibiotique L 17054 (TA3-1)
(Gruppo Lepetit S.p.A.)

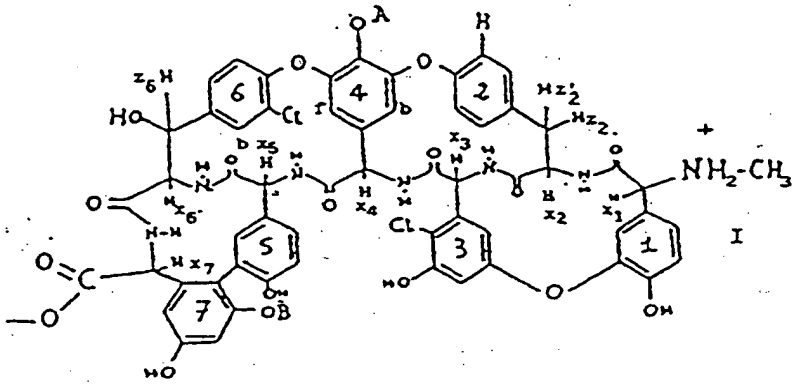
E) R_f de 0,39 dans le système chromatographique suivant :

NaCl 1M contenant 5 g/l de $NaH_2PO_4.H_2O$ 70
acétonitrile 30
ajusté à pH 6,0, en utilisant des plaques de gel de silice 60 silanisé F_{254} Merck (épaisseur de la couche 0,25 mm)
Visualisation :

- Lumière U.V.
- Coloration jaune avec le réactif de Pauly, c'est-à-dire avec l'acide sulfanilique diazoté (J. Chromatog. 20, 171 (1965), Z. Physiol. Chem. 292, 99, (1953))
- Bioautographie en utilisant B. subtilis ATCC 6633 sur milieu minimum de Davis.

F) spectre de masse par bombardement d'atomes rapides (FAB) avec M + H° à environ 1374.

2. Mannosyl aglycone de l'antibiotique A 40926 et ses sels d'addition, qui répondent à la formule suivante, sous la forme de non-sel d'addition :



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dans laquelle
A représente un atome d'hydrogène, et
B représente un groupe mannosyle.

3. Procédé de préparation d'un composé selon la revendication 1 ou 2 qui consiste en l'hydrolyse du complexe de l'antibiotique A 40926 enrichi en facteurs A et B, du facteur A de l'antibiotigue A 40926, du facteur B ou B_0 de l'antibiotique A 40926 ou des mélanges de ceux-ci dans une solution aqueuse concentrée d'un acide fort minéral ou organique, en présence, si on le désire, d'un solvant organique aprotique.

4. Procédé selon la revendication 3, dans lequel l'acide minéral est l'acide sulfurique ou l'acide phosphorique.

5. Procédé selon la revendication 3, dans lequel l'acide organique est l'acide trifluoracétique.

6. Procédé selon la revendication 3, dans lequel les solvants organiques aprotiques sont des éthers alkyliques, alicycliques ou cycliques, des sulfoxydes d'alkyle inférieur ou des amides d'alkyle inférieur.

7. Procédé selon la revendication 6, dans lequel le solvant organique aprotique est le dioxane, le tétrahydrofuranne, le sulfoxyde de diméthyle ou le diméthylformamide.

8. Composé selon la revendication 1 ou 2 pour l'utilisation comme médicament.

9. Utilisation d'un composé selon la revendication 1 ou 2 pour la préparation d'un médicament pour une utilisation antimicrobienne.

10. Composition pharmaceutique comprenant un composé selon la revendication 1 ou 2 en mélange avec un support pharmaceutiquement acceptable.

Rev ndications pour les Etats contractants suivants : AT, GR, ES

1. Procédé de préparation du mannosyl aglycone de l'antibiotique A 40926 et de ses sels d'addition, qui prés nte les caractéristiques suivantes sous la forme non-sel d'addition :

A) spectre d'absorption ultraviolet présentant les maxima d'absorption suivants :

		λ max (nm)
a)	HCl 0,1 N	280
b)	tampon phosphate pH 7,38	280 300 (épaulement)
c)	hydroxyde de potassium 0,1 N	298
d)	tampon phosphate pH 9,0	282 300 (épaulement)

B) spectre d'absorption IR présentant les maxima d'absorption suivants (cm^{-1}) :
3700-3100 ; 3000-2800 (nujol) ; 1655 ; 1620-1540 ; 1505 ; 1460 (nujol) ; 1375 (nujol); 1350-1250 ; 1210 ; 1150 ; 1020; 970 ; 850 ; 810

C) spectre de RMN-^{1}H présentant les groupes de signaux suivants (en ppm) à 270 MHz, enregistrés dans du DMSO d_6 (sulfoxyde d'hexadeutérodiméthyle) plus CF_3COOH en utilisant le TMS comme étalon interne (0,00 ppm), (δ = ppm, multiplicité, attributions) :
2,51, s ($DMSOd_5$) ; 2,50, s (NCH_3) ; 2,88, m (Z2) ; 3,30, m (Z'2) ; 4,08, m (X6) ; 4,44, d (X5) ; 4,49, d (X7) ; 4,83, m (X2) ; 5,02, s (4F) ; 5,08, s (Z6) ; 5,31, s (proton anomère du mannose) ; 5,53, d (X4) ; 5,78, s (4B) ; 6,08, d (X3) ; 7,70, s (6B) ; 6,44÷8,52 (NH aromatiques et peptidiques)

D) temps de rétention (R_t) de 1,18 par rapport à l'antibiotique L 17054 (TA3-1) (R_t = 8,78 min); lorsqu'il est analysé par HPLC en phase inversée dans les conditions suivantes :

Colonne : Ultrasphere® ODS (5 µm) Altex (Beckman) 4,6 mm (d.i.) x 250 mm

Pré-colonne : Brownlee Labs. RP 18 (5 µm)

Eluant A : CH_3CN 10 % } ajusté à
(2,5 g/l) $NaH_2PO_4.H_2O$ 90 % } pH 6,0

Eluant B : CH_3CN 70 % } ajusté à
(2,5 g/l) $NaH_2PO_4.H_2O$ 30 % } pH 6,0

Elution : gradient linéaire de 5 % à 60 % d'éluant B dans l'éluant A, en 40 min.

Débit : 1,6 ml/min

Détecteur UV : 254 nm

Etalon interne : antibiotique L 17054 (TA3-1) (Gruppo Lepetit S.p.A.)

E) R_f de 0,39 dans le système chromatographiqu suivant: NaCl 1M contenant 5 g/l de $NaH_2PO_4.H_2O$ 70
acétonitrile 30
ajusté à pH 6,0, en utilisant d s plaques de gel de silice 60 silanisé F_{254} Merck (épaisseur de la couche 0,25 mm)
Visualisation :
- Lumière U.V.
- Coloration jaune avec le réactif de Pauly, c'est-à-dire avec l'acide sulfanilique diazoté (J. Chromatog. 20, 171 (1965), Z. Physiol. Chem. 292, 99, (1953))
- Bioautographie en utilisant B. subtilis ATCC 6633 sur milieu minimum de Davis.

F) spectre de masse par bombardement d'atomes rapides (FAB) avec M + $H^{\oplus}$ à environ 1374,

qui consiste en l'hydrolyse du complexe de l'antibiotique A 40926 enrichi en facteurs A et B, du facteur A, du facteur B ou du facteur B_0 de l'antibiotique A 40926 ou des mélanges de ceux-ci dans une solution aqueuse concentrée d'un acide fort minéral ou organique, en présence, si on le désire, d'un solvant organique aprotique.

2. Procédé selon la revendication 1, dans lequel l'acide minéral est l'acide sulfurique ou l'acide phosphorique.

3. Procédé selon la revendication 1, dans lequel l'acide organique est l'acide trifluoracétique.

4. Procédé selon la revendication 1, dans lequel les solvants organiques aprotiques sont des éthers alkyliques, alicycliques ou cycliques, des sulfoxydes d'alkyle inférieur ou des amides d'alkyle inférieur.

5. Procédé selon la revendication 4, dans lequel le solvant organique aprotique est le dioxane, le tétrahydrofuranne, le sulfoxyde de diméthyle ou le diméthylformamide.

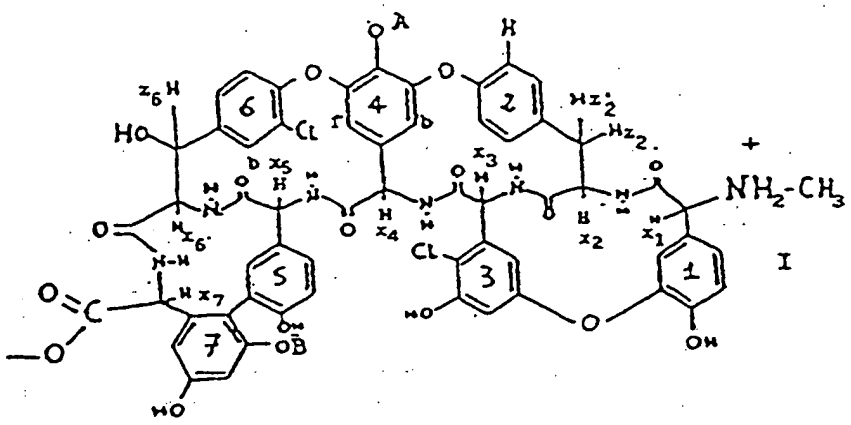
6. Procédé selon la revendication 1, dans lequel la température de réaction est comprise entre 0 °C et la température du reflux.

7. Procédé selon la revendication 6, dans lequel la température de réaction est comprise entre 15 °C et 75 °C.

8. Procédé selon les revendications 1 et 6, dans lequel l'acide fort est l'acide trifluoracétique aqueux à 85-95% et la température de réaction est la température ambiante.

9. Procédé selon la revendication 1, dans lequel l'hydrolyse est effectuée en présence d'un mélange 2:1 à 1:2 d'acide sulfurique aqueux 1-2 N et de dioxane.

10. Procédé selon la revendication 1, pour préparer le mannosyl aglycone de l'antibiotique A 40926 et ses sels d'addition qui répond à la formule suivante, sous la forme de non-sel d'addition :



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dans laquelle

A représente un atome d'hydrogène, et

B représente un groupe mannosyle.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. Antibiotikum A 40926 Mannosylaglycon und dessen Additionssalze, welches in der Nicht-Additionssalzform die folgenden Kennmerkmale besitzt:

A) Ultraviolettabsorptionsspektrum, welches die folgenden Absorptionsmaxima zeigt:

		λ max (nm)
a)	0,1 N HCl	280
b)	Phosphatpuffer pH 7,38	280 300 (Schulter)
c)	0,1 N Kaliumhydroxid	298
d)	Phosphatpuffer pH 9,0	282 300 (Schulter)

B) Infrarotabsorptionsspektrum, welches die folgenden Absorptionsmaxima zeigt (cm^{-1}):
3700-3100; 3000-2800 (Nujol); 1655; 1620-1540; 1505; 1460 (Nujol); 1375 (Nujol); 1350-1250; 1210; 1150; 1020; 970; 850; 810

c) ^{1}H-NMR-Spektrum, welches die folgenden Gruppen von Signalen (in ppm) bei 270 MHz in DMSO d_6 (Hexadeuterodimethylsulfoxid) plus CF_3COOH mit TMS als internem Standard (0,00 ppm) zeigt, (δ = ppm, Multiplizität, Zuordnungen):
2,51, s (DMSO d_5); 2,50, s (NCH_3); 2,88 m (Z2); 3,30, m (Z'2); 4,08, m (X6); 4,44, d (X5); 4,49, d (X7); 4,83, m (X2); 5,02, s (4F); 5,08, s (Z6); 5,31, s (anomeres Proton von Mannose);
5,53, d (X4); 5,78, s, (4B); 6,08, d (X3); 7,70, s (6B); 6,44÷8,52 (aromatische und peptidische NH's)

D) Retentionszeit (R_t) von 1,18 relativ zum Antibiotikum L 17054 (TA3-1) (R_t = 8,78 min) bei Analyse mittels Umkehrphasen-HPLC unter den folgenden Bedingungen:

Säule: Ultrasphere® ODS (5 µm) Altex (Beckman)
4,6 mm (Innendurchmesser) x 250 mm

Vorsäule: Brownlee Labs. RP 18 (5 µm)

Eluierungsmittel A: CH_3CN 10 % } eingestellt
(2,5 g/l) $NaH_2PO_4.H_2O$ 90 % } auf pH 6,0

Eluierungsmittel B: CH_3CN 70 % } eingestellt
(2,5 g/l) $NaH_2PO_4.H_2O$ 30 % } auf pH 6,0

Elution: linearer Gradient von 5 % bis 60 % von Eluierungsmittel B in Eluierungsmittel A, in 40 min

Durchflußgeschwindigkeit: 1,6 ml/min

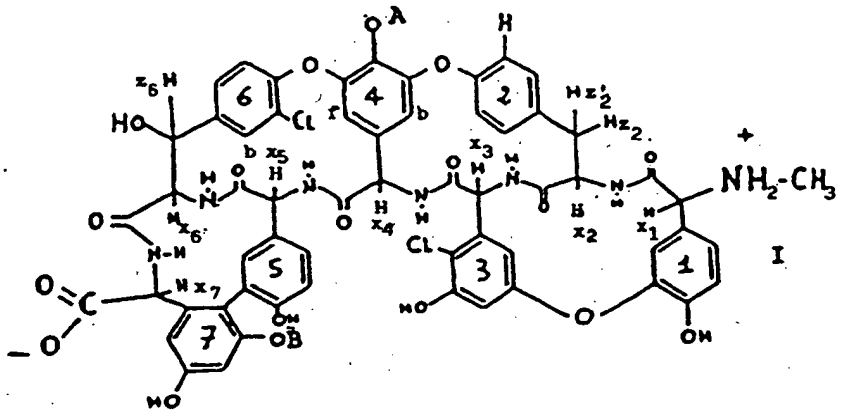
UV-Detektor: 254 nm

Interner Standard: Antibiotikum L 17054 (TA3-1) (Gruppo Lepetit S.p.A.)

E) R_f-Wert von 0,39 im folgenden Chromatographiesystem: 1 M NaCl mit 5 g/l $NaH_2PO_4.H_2O$ 70
Acetonitril 30
eingestellt auf pH 6,0, unter Verwendung von silanisierten Silicagel 60 F_{254} Merck-Platten (Schichtdicke 0,25 mm)
Sichtbarmachung:
- UV-Licht
- Gelbe Färbung mit Pauly-Reagens, d.i. diazotierte Sulfanilsäure (J. Chromatog. 20, 171 (1965), Z. Physiol. Chem. 292, 99 (1953))
- Bioautographie mit B. subtilis ATCC 6633 auf Davis-Minimalnährmedium.

F) Ein fast atom bombardment (FAB)-Massenspektrum mit H + $H^{\oplus}$ bei etwa 1374.

2. Antibiotikum A 40926 Mannosylaglycon und dessen Additionssalze, welches in der Nicht-Additionssalzform die folgende Formel besitzt:



I

worin

A Wasserstoff darstellt und

B Mannosyl bedeutet.

3. Verfahren zur Herstellung einer Verbindung nach Anspruch 1 oder 2, welches das Hydrolysieren von Antibiotikum A 40926-Komplex, welcher mit den Faktoren A und B angereichert ist, von Antibiotikum A 40926 Faktor A, von Antibiotikum A 40926 Faktor B oder B_0 oder von Gemischen hievon in einer konzentrierten wäßrigen Lösung einer starken Mineralsäure oder starken organischen Säure gegebenenfalls in Gegenwart eines aprotischen organischen Lösungsmittels umfaßt.

4. Verfahren nach Anspruch 3, worin die Mineralsäure Schwefelsäure oder Phosphorsäure ist.

5. Verfahren nach Anspruch 3, worin die organische Säure Trifluoressigsäure ist.

6. Verfahren nach Anspruch 3, worin die aprotischen organischen Lösungsmittel alicyclische oder cyclische Alkylether, Niederalkylsulfoxide oder Niederalkylamide sind.

7. Verfahren nach Anspruch 6, worin das aprotische organische Lösungsmittel Dioxan, Tetrahydrofuran, Dimethylsulfoxid oder Dimethylformamid ist.

8. Verbindung nach Anspruch 1 oder 2 zur Verwendung als Arzneimittel.

9. Verwendung einer Verbindung nach Anspruch 1 oder 2 zur Herstellung eines Arzneimittels für den antimikrobiellen Gebrauch.

10. Pharmazeutische Zusammensetzung, umfassend eine Verbindung nach Anspruch 1 oder 2 in Vermischung mit einem pharmazeutisch annehmbaren Träger.

Patentansprüche für folgenden Vertragsstaat : AT, GR, ES

1. Verfahren zur Herstellung von Antibiotikum A 40926 Mannosylaglycon und dessen Additionssalzen, welches in der Nicht-Additionssalzform die folgenden Kennmerkmale aufweist:

A) Ultraviolettabsorptionsspektrum, welches die folgenden Absorptionsmaxima zeigt:

		λ max (nm)
a)	0,1 N HCl	280
b)	Phosphatpuffer pH 7,38	280 300 (Schulter)
c)	0,1 N Kaliumhydroxid	298
d)	Phosphatpuffer pH 9,0	282 300 (Schulter)

B) Infrarotabsorptionsspektrum, welches die folgenden Absorptionsmaxima zeigt (cm^{-1}):
3700-3100; 3000-2800 (Nujol); 1655; 1620-1540; 1505; 1460 (Nujol); 1375 (Nujol); 1350-1250; 1210; 1150; 1020; 970; 850; 810

C) ^{1}H-NMR-Spektrum, welches die folgenden Gruppen von Signalen (in ppm) bei 270 MHz in DMSO d_6 (Hexadeuterodimethylsulfoxid) plus CF_3COOH mit TMS als internem Standard (0,00 ppm) zeigt, (δ = ppm, Multiplizität, Zuordnungen):
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Acetonitril 30
eingestellt auf pH 6,0, unter Verwendung von silanisierten Silicagel 60 F_{254} Merck-Platten

(Schichtdicke 0,25 mm)
Sichtbarmachung:
- UV-Licht
- Gelbe Färbung mit Pauly-Reagens, d.i. diazotierte Sulfanilsäure (J. Chromatog. 20, 171 (1965), Z. Physiol. Chem. 292, 99 (1953))
- Bioautographie mit B. subtilis ATCC 6633 auf Davis-Minimalnährmedium.

F) Ein fast atom bombardment (FAB)-Massenspektrum mit M + $H^{\oplus}$ bei etwa 1374,

welches das Hydrolysieren von Antibiotikum A 40926-Komplex, welcher mit den Faktoren A und B angereichert ist, von Antibiotikum A 40926 Faktor A, von Antibiotikum A 40926 Faktor B oder B_0 oder von Gemischen hievon in einer konzentrierten wäßrigen Lösung einer starken Mineralsäure oder starken organischen Säure gegebenenfalls in Gegenwart eines aprotischen organischen Lösungsmittels umfaßt.

2. Verfahren nach Anspruch 1, worin die Mineralsäure Schwefelsäure oder Phosphorsäure ist.

3. Verfahren nach Anspruch 1, worin die organische Säure Trifluoressigsäure ist.

4. Verfahren nach Anspruch 1, worin die aprotischen organischen Lösungsmittel alicyclische oder cyclische Alkylether, Niederalkylsulfoxide oder Niederalkylamide sind.

5. Verfahren nach Anspruch 4, worin das aprotische organische Lösungsmittel Dioxan, Tetrahydrofuran, Dimethylsulfoxid oder Dimethylformamid ist.

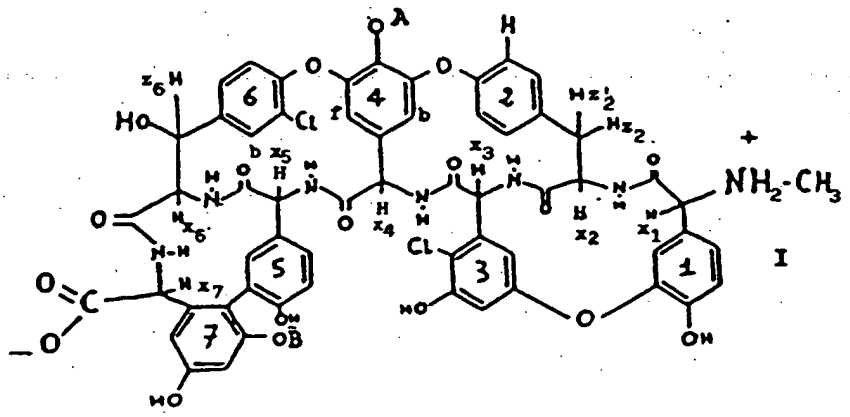
6. Verfahren nach Anspruch 1, worin die Reaktionstemperatur von 0° C bis zur Rückflußtemperatur beträgt.

7. Verfahren nach Anspruch 6, worin die Reaktionstemperatur von 15° C bis 75° C beträgt.

8. Verfahren nach Anspruch 1 und 6, worin die starke Säure 85-95 %ige wäßrige Trifluoressigsäure ist und die Reaktionstemperatur Raumtemperatur ist.

9. Verfahren nach Anspruch 1, worin die Hydrolyse in Gegenwart eines Gemisches von 2:1 bis 1:2 von wäßriger 1-2N Schwefelsäure und Dioxan ausgeführt wird.

10. Verfahren nach Anspruch 1 zur Herstellung von Antibiotikum A 40926 Mannosylaglycon und dessen Additionssalzen, welches in der Nicht-Additionssalzform die folgende Formel besitzt:

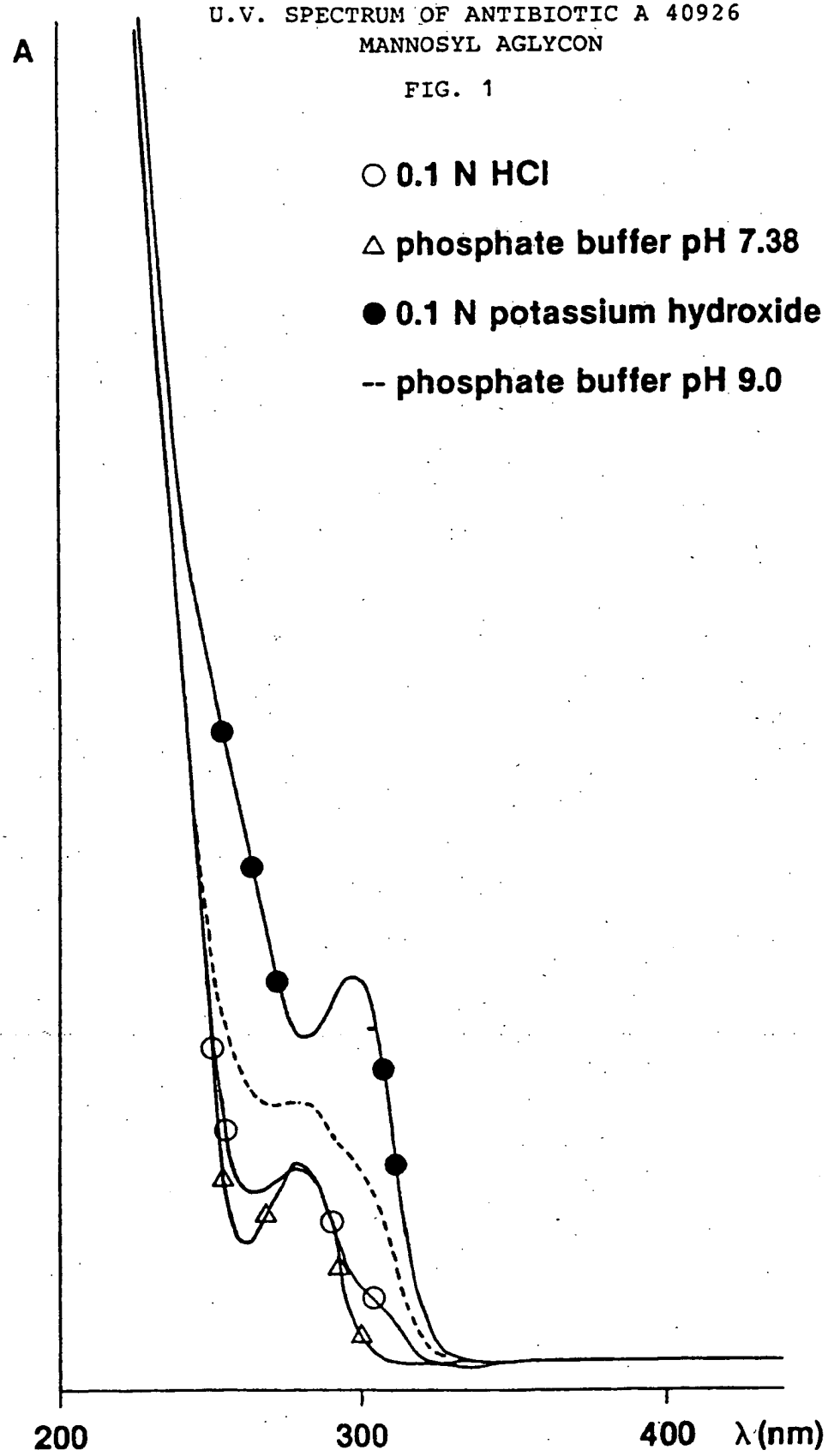


I

worin
A Wasserstoff darstellt und
B Mannosyl bedeutet.

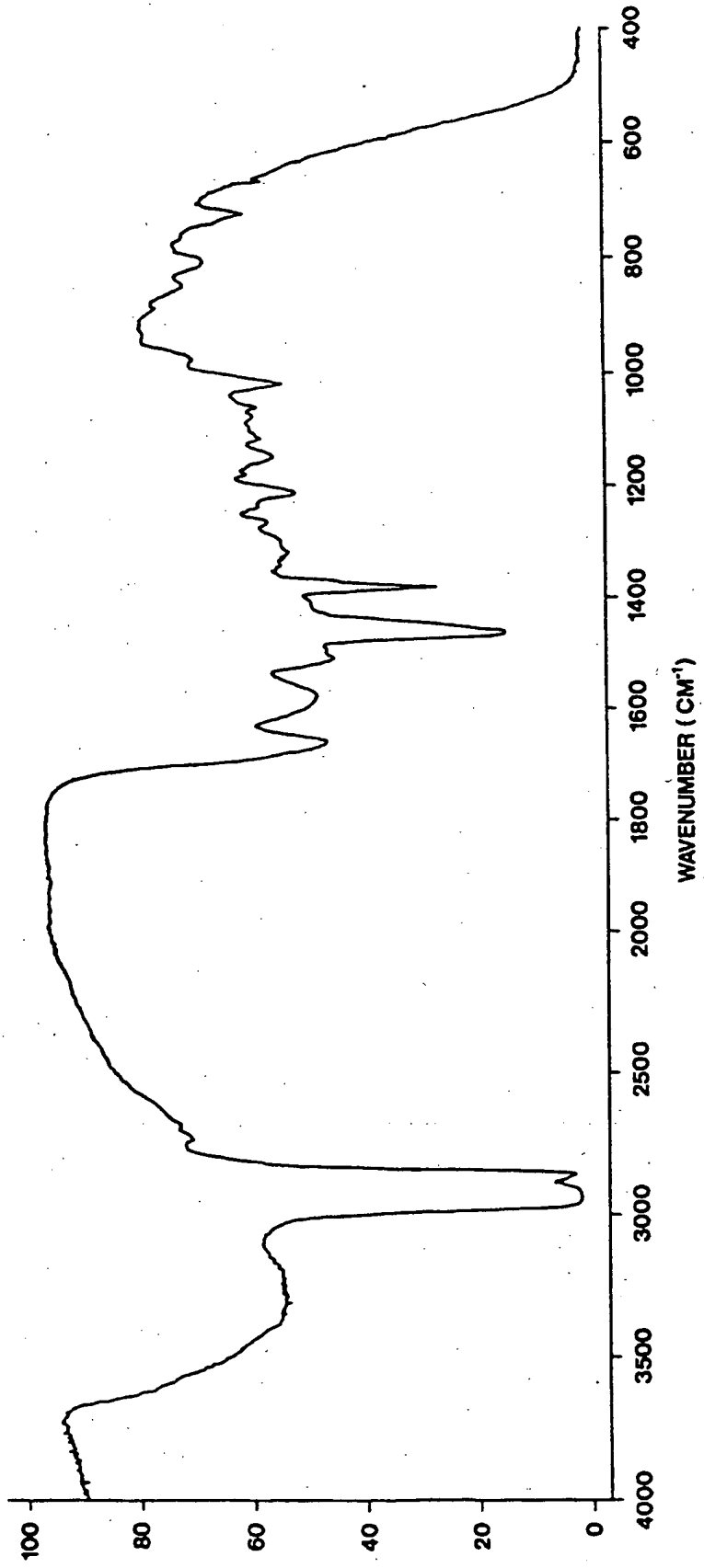
U.V. SPECTRUM OF ANTIBIOTIC A 40926
MANNOSYL AGLYCON

FIG. 1



I.R. SPECTRUM OF ANTIBIOTIC A 40926
MANNOSYL AGLYCON

FIG. 2



^{1}H-N.M.R. SPECTRUM OF ANTIBIOTIC A 40926
MANNOSYL AGLYCON

FIG. 3